

# Immunomagnetic Enrichment, Genomic Characterization, and Prognostic Impact of Circulating Melanoma Cells

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## ABSTRACT

**Purpose:** The finding of melanoma cells in the peripheral blood, thus far mainly inferred from the PCR-based demonstration of tyrosinase mRNA, has been associated with metastatic melanoma. Neither the malignant nature nor the prognostic significance of circulating cells could be established. To address this question, we analyzed immunomagnetically isolated circulating melanoma cells for chromosomal aberrations and performed a clinical follow-up study of the enrolled patients.

**Experimental Design:** In a prospective study, blood samples were taken from 164 melanoma patients and 50 donors without malignant disease. Circulating melanoma cells were enriched by immunomagnetic cell sorting using a murine monoclonal antibody against the melanoma-associated chondroitin sulfate proteoglycan. To prove the malignant origin of the positive cells and to define their chromosomal aberrations, we analyzed the genomes of 15 individually isolated cells from seven patients by single-cell comparative genomic hybridization (SCOMP).

**Results:** Absolute and relative frequencies of circulating melanoma cells were associated with stage and with the presence or absence of detectable tumor. The detection of two or more cells correlated significantly with a reduced survival of patients with metastatic melanoma. All of the cells that were analyzed by SCOMP displayed multiple

chromosomal changes and carried aberrations typical for melanoma.

**Conclusions:** Immunomagnetic enrichment enables isolation and genomic characterization of circulating melanoma cells. The prognostic impact on survival of metastatic patients apparently reflects the aggressiveness of an ongoing tumor spread. Direct genomic analysis of the enriched and isolated cells will help to clarify the molecular-genetic basis of the establishment of generalized melanoma.

## INTRODUCTION

A locally confined tumor progresses to systemic metastasis by clonal expansion of tumor cells, which are selected for specific biological characteristics such as dissemination and ectopic survival. Animal studies with highly aggressive cultured melanoma cells have shown that 99.9% of intravascularly injected tumor cells will fail to found a metastasis (1). Consequently, both the quantity and the quality (2) of the very tumor cells that invade the lymphatic or blood vessels seem to determine metastatic success and hence the fate of the individual patient, necessitating the direct investigation of circulating tumor cells rather than the sessile cells of the primary tumor. Unfortunately, the search for circulating melanoma cells among millions of blood cells is very cumbersome. Thus far, numerous reverse transcription (RT)-PCR-based protocols were developed to detect specific tumor-associated transcripts in mRNA preparations from peripheral blood, although such approaches permit neither exact quantification nor genotypic or phenotypic characterization of circulating tumor cells (3). Moreover, using RT-PCR, one cannot even determine whether circulating tumor cells are detected rather than circulating nucleic acids, a difference that is obviously relevant for the question of metastasis formation.

Recently we developed a cellular approach for the detection of intact circulating melanoma cells using a monoclonal antibody against the melanoma-associated chondroitin sulfate proteoglycan (MCSP) and magnetically activated cell sorting. The assay allows the detection, enumeration, and further characterization of the isolated cells. We demonstrated that the sensitivity of the assay is in the range of the data published for RT-PCR and that MCSP-positive melanoma cells can be detected in the peripheral blood of patients with advanced melanoma (4, 5). In a novel, prospective study of chemo-naïve melanoma patients we have now applied the method with minor modifications to blood samples from 164 melanoma patients to determine a possible correlation with clinical stage, with the presence of manifest disease, and with the survival of the patients. We then applied a recently developed amplification method for single-cell DNA (6) after enrichment of single circulating melanoma cells. The finding of genomic aberrations by comparative genomic hybridization (CGH) in circulating

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MCSP-positive cells proves their malignant origin and opens the door to study the genetics of disseminating melanoma.

## MATERIALS AND METHODS

**Patient Selection.** Between June 1997 and November 2000, 164 patients presenting to our clinic were enrolled in the study. Inclusion criteria comprised a histologically documented diagnosis of cutaneous or uveal melanoma with either localized or metastatic disease. Exclusion criterion was a history of chemotherapy or immunotherapy. Fifty-nine patients had localized disease and were examined either before surgical removal of the primary tumor (American Joint Committee on Cancer stage I and II with evidence of disease) or at least 72 h after complete surgery (without evidence of disease). Forty-two patients had regional nodal involvement and were examined before surgery for regional metastases (American Joint Committee on Cancer stage III with evidence of disease) or after complete resection of regional metastases (without evidence of disease). Sixty-three patients were American Joint Committee on Cancer stage IV melanoma, including patients with distant metastases (with evidence of disease) and patients without evidence of disease after surgery for single organ metastases. Seventy-nine patients were female, and 85 patients were male. The mean age was 54 years (range, 17–85 years). All of the patients were treated and followed up at the Department of Dermatology, University of Tübingen, Germany. Clinical stage was defined according to the American Joint Committee on Cancer 1992 guidelines (7). Follow-up data were available for 151 patients. Additionally, blood samples from 50 healthy donors or patients with non-melanoma disease were examined.

**Blood Samples.** After informed consent was obtained, 50 ml of heparinized peripheral blood was collected from the patients. The blood samples were processed immediately. A second blood sample was taken from six patients with distant metastases and from one patient with regional metastases for CGH analysis. For statistical analysis, only the first blood sample was evaluated.

**Immunomagnetic Melanoma Cell Enrichment (IMCE).** Enrichment was performed as described previously with the modification that a smaller column (MS+ instead of a LS+ column) was used to minimize melanoma cell loss during the enrichment procedure (4). Briefly, after density gradient centrifugation of 50 ml of heparinized peripheral venous blood, the interphase was incubated with the murine monoclonal antibody 9.2.27. Monoclonal antibody 9.2.27 (isotype IgG2a) is directed against a  $M_r$  250,000 human MCSP (8). Purified, lyophilized antibody was kindly provided by Dr. R. A. Reisfeld (Department of Immunology, The Scripps Research Institute, La Jolla, CA 92037) and was used at a final concentration of 5  $\mu$ g/ml. Positive cells were labeled with 100  $\mu$ l of goat-antimouse microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany) and were enriched by magnetically activated cell sorting by passage over an MS+ separation column. (Miltenyi Biotec). Cells of the positive fraction were attached to poly-L-lysine-covered glass slides (Sigma, Deisenhofen, Germany) and were stained using the alkaline phosphatase-antialkaline phosphatase technique (APAAP), München, Germany) as a substrate and Mayer's hemalaun (Merck, Darmstadt, Germany) for counterstaining.

For genomic analysis of individual circulating tumor cells, a second blood sample was drawn. After enrichment cells were immobilized on positively charged slides and were stained by 5-bromo-4-chloro-3-indoyl phosphate and Nitroblue tetrazolium (BCIP/NBT) as substrate for the alkaline phosphatase as described previously (9, 10). In these cases, counterstaining was omitted.

**Evaluation Criteria.** Slides were analyzed under a Zeiss light microscope. Neofuchsin and counterstained cells were judged to be positive only when they showed bright red immunostaining on at least 50% of the cell surface and had the morphological characteristics of tumor cells (size and shape of the cell and nucleus). In all cases, the entire positive fraction of a blood sample was examined without knowledge of the clinical stage of the patient. The sample was considered positive if one or more positive cells were found. The absolute number of cells detected per sample was recorded.

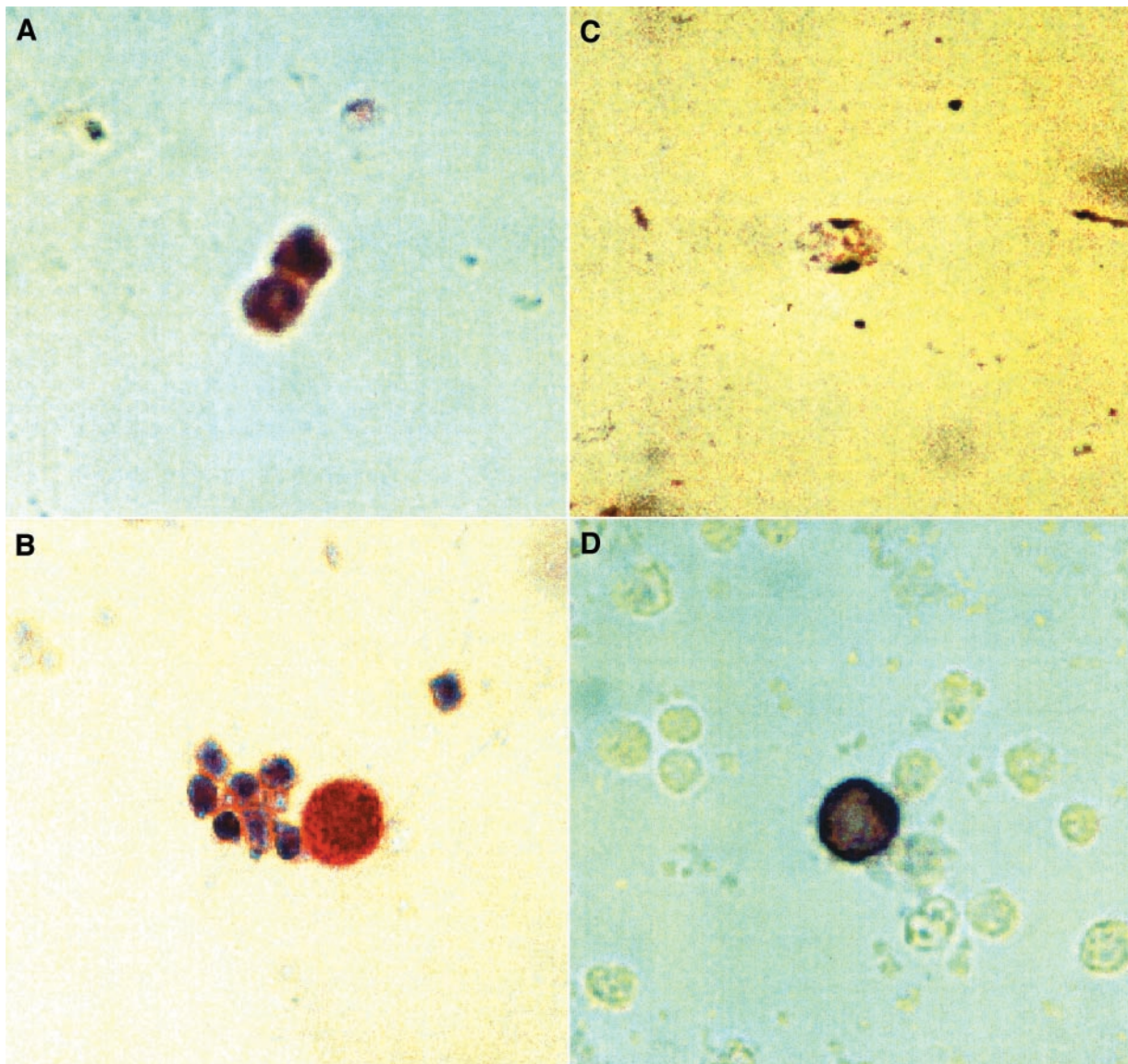
**Single-Cell CGH.** Positive cells were isolated by micro-manipulation and single-cell CGH was performed as published by Klein *et al.* (6) with the modifications described by Klein *et al.* in Ref. 8. In brief, after isolation, proteinase K served to digest cellular proteins, the single-cell genome was digested using the restriction enzyme *MseI*, adaptors were ligated to the 5' overhangs, and the DNA fragments were amplified by PCR, resulting in a *MseI* representation of a single-cell genome. These amplicons were labeled and hybridized. Cluster analysis was performed using the hierarchical clustering (average linkage mode) by M. Eisen as described in Klein *et al.* (9).

**Statistical Analysis.** Spearman's rank correlation coefficient was used to demonstrate the relationship between clinical stage and detection of melanoma cells. Correlation of melanoma cells in the blood and evidence of disease was assessed using Pearson's  $\chi^2$  test. Overall survival was calculated from the time of inclusion in the study until death and analyzed by Kaplan-Meier estimates. Survival curves were compared using a log-rank test. The independent prognostic value of IMCE positivity in the stages IIIB and IV, of evidence of disease, and of stepwise-coded IMCE positivity (negative *versus* 1 *versus* >1) was assessed by Cox's proportional hazard regression. Variables included in the analysis were the clinical stage (distant *versus* regional metastases) and IMCE positivity. Proportionality assumption was tested by evaluation of Schoenfeld residuals. In all statistical tests, a *P* of <0.05 was considered statistically significant. Statistical analysis was performed using SPSS statistical software (SPSS for Windows, Release 10.0, 2000, Chicago, IL) and the R statistical computing package (The R Foundation for Statistical Computing, Vienna, Austria).

## RESULTS

Circulating melanoma cells were detected in 43 (26%) of the 164 melanoma patients examined, and control samples of 50 non-melanoma patients proved to be negative. The positive cells showed features typical of malignant cells (Fig. 1B). These cells were larger than the negative mononuclear cells and showed nearly 100% bright red staining of their cell membrane. In some patients, the tumor cells contained large numbers of melanin granules. A high nuclear:cytoplasmic ratio was often associated with prominent nucleoli. In all cases, only single cells were





**Fig. 1** Melanoma-associated chondroitin sulfate proteoglycan (MCSP)-positive cells stained with neofuchsin and hemalaun (A and B) and with BCIP/NBT (C and D). The strong signal of the melanoma cell (B) can be easily differentiated from that of granulocytes (A), which show the characteristic polymorphonuclear morphology. Strong staining intensity identifies melanoma cells using BCIP/NBT as substrate. C, weakly stained granulocytes; D, melanoma cell.

detected, except for one sample containing a three-cell cluster. We also noted a very faint staining on a few granulocytes. However, by applying our evaluation criteria, we could safely exclude them from analysis (Fig. 1A). In positive patients, 1–21 cells were detected in stage I/II patients (median stage I, 1; median stage II, 3), 1–456 cells (median, 2) were detected in stage III patients, and 1–2410 cells (median, 5) were detected in stage IV patients. Thus, the number of cells detected varied widely. Application of a categorical test, such as the  $\chi^2$  test, demonstrated significant differences for positivity between low- (I, II) and high-grade stages (III, IV) of disease ( $P < 0.001$ ). The positive finding correlated significantly with the clinical stage of the patient ( $r = 0.481$ ;  $P < 0.001$ ), with 5 (8%) of 59, 11 (26%)

of 42, and 27 (43%) of 63 patients being positive in stage I/II, stage III, and stage IV, respectively (Table 1).

Next, the relationship between detectable tumor and the finding of MCSP-positive cells was evaluated. Clinical manifestation of disease was recorded as no evidence of disease (NED) or evidence of disease (ED). No-evidence-of-disease patients were defined as patients without primary tumor or metastases when the blood samples were drawn. Patients were classified as having evidence of disease when the blood sample was taken before removal of the primary melanoma, regional lymph node, or distant metastases. One hundred seven (65%) of the 164 patients showed evidence of disease, whereas 57 (35%) patients were without evidence of disease. The correlation be-

tween the presence of detectable tumor and a positive IMCE finding was statistically significant ( $P < 0.001$ ; Table 2) regardless of disease stage. Only 1 (2%) of the 57 patients without evidence of disease had circulating melanoma cells, whereas 42 (39%) of the 107 patients with evidence of disease tested positive.

Furthermore, we examined the impact of circulating melanoma cells on survival for patients with metastatic melanoma (stages III, IV). Statistical analysis for stage I/II patients was not performed because of the low number of recurrences and deaths in this subgroup. Whereas the mere presence of circulating melanoma cells was not associated with a significantly decreased overall survival for patients with metastatic melanoma a statistically significant reduction in survival time was observed when we detected more than one positive cell (Fig. 2;  $P = 0.001$ ). Median survival for 26 patients with two or more tumor cells in peripheral blood was 7 months in comparison with 28 months for 66 patients with less than two circulating cells. Although distant metastasis (stage IV) imposed a high risk to die from melanoma (hazard ratio, 4.25), Cox regression analysis revealed that detection of melanoma cells in the peripheral blood conferred a 2-fold risk on patients to die within the observation time, independent of the presence of distant metas-

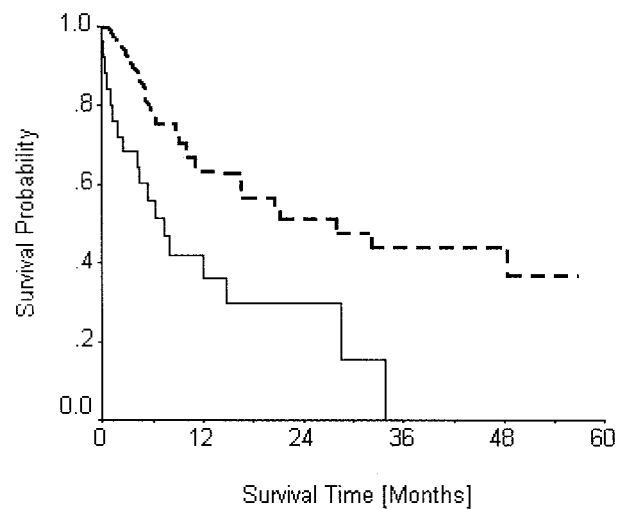


Fig. 2 Kaplan-Meier survival curves of stage III and IV patients [dashed line, detection of less than two cells by immunomagnetic melanoma cell enrichment (IMCE); thin solid line, detection of two or more cells by IMCE;  $P = 0.001$ ].

Table 1 Immunomagnetic melanoma cell enrichment (IMCE) in correlation with the clinical stage of the patients

	Total No.	IMCE positive		Median MSCP <sup>a</sup> cell count of positive samples (range)
		No.	%	
Stage I	29	2	7	1 (1–11)
Stage II	30	3	10	3 (1–21)
Stage III	42	11	26	2 (1–456)
Stage IV	63	27	43	5 (1–2410)

MSCP, melanoma-associated chondroitin sulfate proteoglycan; No., number of examined patients.

Table 2 Immunomagnetic detection of circulating melanoma cells in correlation with clinical evidence of disease of the patient

	Total no. <sup>a</sup>	IMCE positive	
		no.	%
Stage I			
ED	13	2	15
NED	16	0	0
Stage II			
ED	11	3	27
NED	19	0	0
Stage III			
ED	24	10	42
NED	18	1	6
Stage IV			
ED	59	27	46
NED	4	0	0
Total			
ED	107	42	39
NED	57	1	2

<sup>a</sup> no., number of examined patients; ED, evidence of disease; NED, no evidence of disease; IMCE, immunomagnetic melanoma cell enrichment.

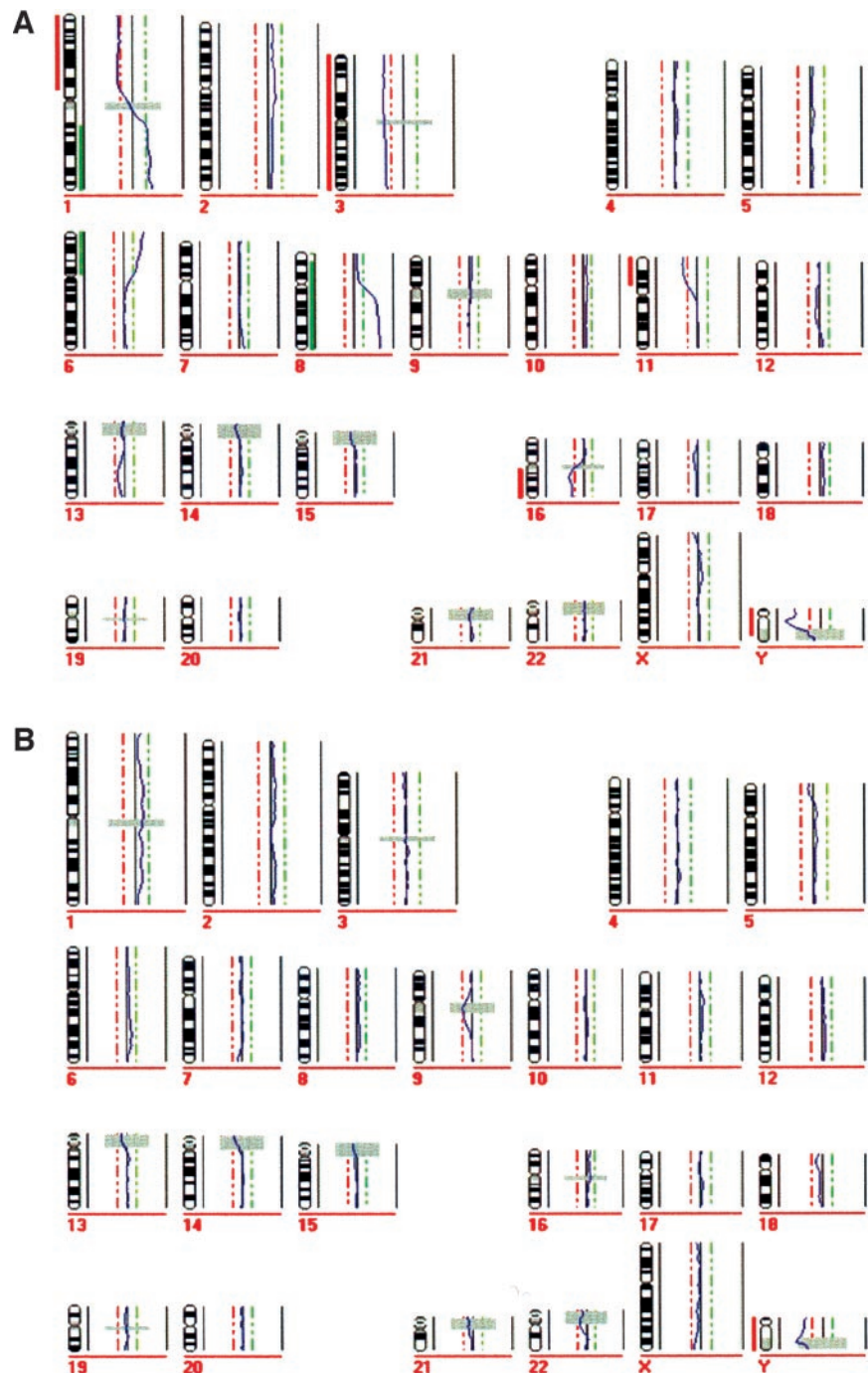
Table 3 Cox proportional hazards model

Factor	Hazard ratio [95% confidence interval]	$P$
Distant vs. regional metastasis	4.25 [1.9–9.4]	<0.001
IMCE <sup>a</sup> $\geq$ 1 cells vs. 0 cells	1.2 [0.4–4.0]	0.77
IMCE $\geq$ 2 cells vs. <2 cells	2.0 [1.1–3.7]	0.03
ED vs. NED	3.0 [0.7–11.5]	0.13

<sup>a</sup> IMCE, immunomagnetic melanoma cell enrichment; ED, evidence of disease; NED, no evidence of disease.

tases (Table 3). The presence or absence of detectable tumor in stage III/IV disease had no significant impact on survival ( $P < 0.13$ ).

The significant influence on survival, even in late-stage patients, clearly raises the question about the nature of circulating melanoma cells. A major advantage of cell-based approaches over RT-PCR methods is that the isolated cells can be enumerated as well as further characterized. Genetic profiling of the isolated cells not only allows the demonstration of their malignant origin but may also reveal the metastatic potential of the circulating cells. We, therefore, applied single-cell DNA amplification and CGH to 15 cells from seven patients with stage III and IV disease that had been stained with the alkaline phosphate substrate BCIP/NBT (Fig. 1D). CGH allows screening of the whole single-cell genome for chromosomal aberrations and, therefore, is best suited for testing cells with unknown genetic changes. All of the analyzed cells harbored DNA copy number changes (Fig. 3A and Fig. 4), whereas all simultaneously isolated single cells ( $n = 7$ ) that were not stained by the MSCP antibody displayed balanced CGH profiles (Fig. 3B). In five of six cases with more than one isolated cell from an individual patient, the cells showed clonal relationship as revealed by hierarchical cluster analysis. In these cases, the applied algorithm grouped the cells from one individual patient



**Fig. 3** Comparative genomic hybridization (CGH) profiles of a single circulating melanoma-associated chondroitin sulfate proteoglycan (MCSP)-positive uveal melanoma cell (*A*) and of a single MCSP-negative control cell (*B*). To be considered significant, deviations of the blue result line from the central line had to cross the green (chromosomal gain) or red (chromosomal loss) punctate line. Regions of chromosomal losses (*red*) and gains (*green*) are indicated by colored bars next to the ideogram of each chromosome. The gray shaded horizontal bars indicate regions excluded from analysis because of the prevalence of heterochromatic DNA. Note that the blue line representing the ratio of test and control DNA never crosses the dotted thresholds in the case of the MCSP-negative cell but marks, for example, a complete loss of chromosome 3 in the uveal melanoma cell.

that displayed similar genomic changes in a branch of the dendrogram (Fig. 4).

## DISCUSSION

This study describes a novel access to the, thus far, enigmatic stage of cell dissemination in malignant melanoma. To achieve quantification and characterization of melanoma cells in peripheral blood, we combined the detection of intact circulating melanoma cells by immunomagnetic cell sorting with subse-

quent genomic analysis. Previously, several studies used PCR-based methods to detect circulating melanoma cells, resulting in more than 120 publications (3). Because of the diversity of the different methods, intrinsic problems such as illegitimate expression on mRNA level, and low interlaboratory reproducibility, tumor cell detection by RT-PCR has recently been questioned for clinical use (11).

Immunomagnetic enrichment of melanoma cells in our study is based on the differential expression of the human



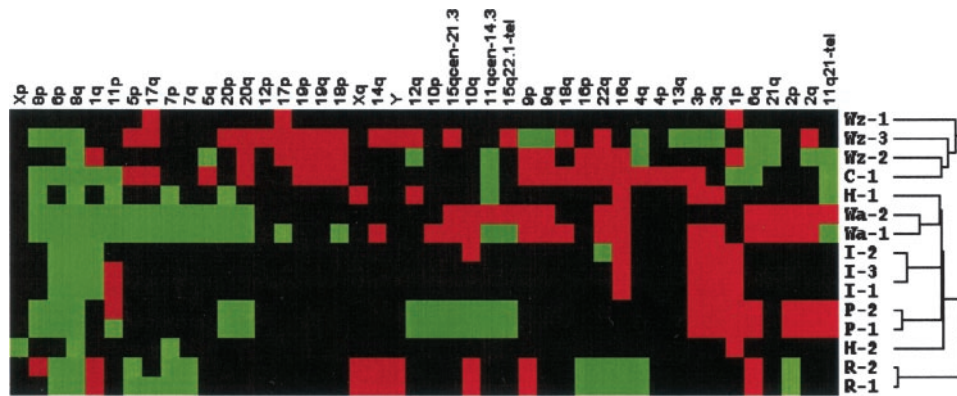


Fig. 4 Cluster analysis of chromosomal aberrations of 15 circulating melanoma-associated chondroitin sulfate proteoglycan (MCSP)-positive tumor cells. The cells were isolated from seven patients with uveal and cutaneous melanomas. The cells are grouped according to the similarity of their chromosomal aberrations by hierarchical clustering. In the cell identifier (on the right side of the image), letters indicate the patient, and numbers indicate the number of the individual cells.

melanoma-associated chondroitin sulfate proteoglycan, which is uniformly expressed on >90% of human melanoma tissues and cell lines (8, 12, 13). The applied monoclonal antibody 9.2.27 has recently been used for tumor cell detection in peripheral blood, but positive findings were not validated on a genomic level (14). Despite the alleged exclusive melanocytic expression of this proteoglycan antigen (8), we noted a very faint staining on few granulocytes in some samples. However, we were able to define precise evaluation criteria to allow unambiguous melanoma cell identification. Using neofuchsin as substrate of alkaline phosphatase in combination with conventional nuclear staining such as hematoxylin, we can clearly differentiate the faintly positive granulocytes from melanoma cells by staining intensity and characteristic nuclear morphology. For the genomic analysis, the intense color obtained with the substrate BCIP/NBT substrate gave sufficient certainty on the melanomic origin of the stained cells (Fig. 1D). In contrast to neofuchsin, this BCIP/NBT does not damage the DNA but precludes subsequent nuclear counterstaining. The identity of the stained cells as melanoma cells was unambiguously proven by subsequent genome analysis, which revealed multiple chromosomal changes in each instance.

We found a statistically significant association between the detection of circulating tumor cells and tumor stage as well as the presence of detectable tumor. Also, the detection of circulating melanoma cells in 5 of the 24 patients with localized disease (stage I, II) before surgery of the primary tumor indicates that the presence of small tumor masses is, in some cases, sufficient for tumor cell detection in peripheral blood. Setting a threshold at two circulating melanoma cells, we can assign a 2-fold risk for MCSP-positive patients to die from melanoma within the observation period independent from whether distant metastasis is already established or whether the disease is locally confined to lymph nodes. The finding that the threshold of two or more cells had an impact on survival in contrast to the presence *versus* the absence of tumor cells in the peripheral blood apparently reflects either the erroneous count of some cells or the stochastic nature of metastasis formation. To address this question, more genomic data of circulating melanoma cells

may enable a determination of their malignant potential. However, it should be noted that the effect of tumor cell counts in bone marrow on the prognostic significance has been observed before (13).

The prognostic impact for advanced tumor stages and the now possible genomic characterization of circulating melanoma cells by single-cell CGH may have important implications for melanoma biology. Obviously, the aggressiveness of advanced metastatic melanomas appears to be reflected by the detection of circulating melanoma cells in the peripheral blood after immunomagnetic enrichment. In addition to proving that MCSP-positive cells are indeed melanoma cells, single-cell CGH now allows genetic dissection of the mutations that are associated with this behavior. Perhaps, the most convincing argument for their identity as melanoma cells is the frequent monosomy of chromosome 3 of the circulating uveal melanoma cells, which has been shown to be the most significant predictor of poor prognosis in uveal melanoma (Figs. 3A and Fig. 4; Refs. 16, 17). Likewise, the loss of chromosomes 6q, 9p, and 10q as well as a gain of chromosome 8q (some of the commonly changed regions in cutaneous melanoma) were often detected in patients with cutaneous melanoma (18, 19). These findings prove their malignant nature and melanoma origin and stand in sharp contrast to the balanced CGH profiles from single normal cells (Fig. 3B). Interestingly, for five of the six patients with more than one tumor cell in the peripheral blood, we could demonstrate clonal relatedness when we performed hierarchical cluster analysis (Fig. 4) of the isolated cells as described previously (9).

Our study has several limitations. We do not yet have enough data to test whether the finding of circulating melanoma cells in stage I and stage II patients will predict clinical outcome, although the results from metastatic patients suggest such a correlation. For prognostic impact, it will be important to know whether the mere presence of circulating tumor cells implies their metastatic outgrowth or whether the risk of distant metastasis formation will be determined by specific genetic aberrations. Immunomagnetic enrichment can easily be applied in every laboratory; however, metaphase CGH is more demanding. For the latter, the rapidly evolving genomic profiling techniques

on DNA microarrays (20, 21) will soon enable the automated genotyping of single circulating melanoma cells. Specific genomic patterns of immunomagnetically enriched cells may predict which cells will grow into distant metastases. Combining the genomic data with gene expression profiling of those cells (22), one may find the answers to many more questions of melanoma cell biology that might direct future staging or patient stratification and the development of novel therapies.

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